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(54) Title: PIGGYBAC TRANSFORMATION SYSTEM

(57) Abstract: The present invention is directed to a transformation system for making transgenic organisms that includes a vector containing a modified *piggyBac* transposon into which is inserted an enhanced green fluorescent protein gene linked to a polyubiquitin promoter sequence and a nuclear localizing sequence; and a helper transposase vector that includes an *hsp70* promoter sequence upstream of the putative *piggyBac* promoter that increases the transformation frequency of this system.

## PIGGYBAC TRANSFORMATION SYSTEM

## BACKGROUND OF INVENTION

Field of the Invention

This invention relates to a transformation system that includes a gene transfer vector containing a modified *piggyBac* transposon (pB) and having the insertion of a marker construct containing an enhanced green fluorescent protein gene (EGFP) linked to a polyubiquitin promoter gene and a nuclear localizing sequence. The invention further relates to a helper vector containing a heat shock protein gene and to methods for using this system to transform eukaryotic cells as well as transgenic organisms produced using the system, especially insect cells and insects, respectively.

Description of the Related Art

The *piggyBac* transposable element from the cabbage looper moth, *Trichoplusia ni* (Cary et al., Virology, Volume 161, 8-17, 1989) has been shown to be an effective gene-transfer vector in the Mediterranean fruit fly, *Ceratitidis capitata* (Handler et al., Proc. Natl. Acad. Sci. USA, Volume 95, 7520-7525, 1998). Use of an unmodified transposase helper under *piggyBac* promoter regulation indicates that *piggyBac* retains autonomous function in the medfly, since transcriptional regulation was maintained, as well as enzymatic activity. This observation was unique since all other successful insect germline transformations had been limited to dipteran species using vectors isolated from the same or another dipteran. The initial transformation of medfly (Loukeris et al., Science, Volume 270, 2002-2005, 1995) used the *Minos* vector from *Drosophila hydei* (Franz & Savakis, Nucl. Acids Res., Volume 19, 6646, 1991), and *Aedes aegypti* has been transformed from *Hermes* (Jasinskiene et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3743-3747, 1998) from *Musca domestica* (Warren et al., Genet. Res. Camb., Volume 64, 87-97, 1994) and *mariner* (Coates et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3748-3751, 1998) from *Drosophila mauritiana* (Jacobson et al., Proc. Natl. Acad. Sci.

USA, Volume 83, 8684-8688, 1986). *Drosophila melanogaster* has been transformed as well by *Hermes* (O=Brochta et al., Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996) *mariner* (Lidholm et al., Genetics, Volume 134, 859-868, 1993), *Minos* (Franz et al., Proc. Natl. Acad. Sci. USA, Volume 91, 4746-4750, 1994) and by the *P* and *hobo* transposons originally discovered in its own genome (Rubin and Spradling, 1989; Blackman et al., EMBO J., Volume 8, 211-217, 1989). *Drosophila virilis* also has been transformed by *hobo* (Lozovskaya et al., Genetics, Volume 143, 365-374, 1995; Gomez & Handler, Insect Mol. Biol., Volume 6, 1-8, 1997) and *mariner* (Lohe et al., Genetics, Volume 143, 365-374, 1996). While the restriction to dipteran vectors is due in part to the limited number of transposon systems available from non-dipteran species, phylogenetic limitations on transposon function is not unexpected considering the deleterious effects functional transposons may have on a host genome. This is, indeed, reflected by the high level of regulation placed on transposon movement among species, among strains within a host species, and even among cell types within an organism (Berg & Howe, *Mobile DNA*, American Society for Microbiology, Washington, D.C. 1989).

The ability of *piggyBac* to function in several dipteran species will be supportive of its use in a wider range of insects, if not other organisms. Most other vector systems function optimally, or have been only tested with their helper transposase under *hsp70* promoter regulation. The transposition efficiency of most vectors has been also found to be influenced by the amount of internal DNA inserted, the position of this DNA within the vector, and the amount of subterminal DNA remaining in the vectors.

The widespread use of *piggyBac* will be limited by the availability of easily detectable and unambiguous transformant markers. Most *Drosophila* transformations, as well as the few nondrosophilid transformations reported have depended on transformant selection by rescue of a mutant visible phenotype, usually eye pigmentation (Ashburner et al., Insect Mol. Biol., Volume 7, 201-213, 1998). Unfortunately, most insect species have neither visible mutant strains, nor the cloned DNA for the wild

type allele of the mutation, and these species require use of new dominant-acting marker genes that confer, preferably, a visible phenotype.

The present invention, discussed below, provides a system that includes vectors for transforming eukaryotic cells, derived from *piggyBac* transposons that are different from related art vectors. Furthermore, the present invention increases the transformation frequency by about eight-fold compared to other *piggyBac* transformation systems.

#### Summary of the Invention

It is therefore an object of the present invention to provide a transformation system contains a vector that includes DNA derived from a *piggyBac* transposon element that allows for the almost precise excision of at least a second DNA sequence that is heterologous and included in the construct and insertion of at least said second heterologous DNA sequence into eukaryotic cells after introduction of the transformation construct containing said first and at least a second DNA into said cell that is then used to form a transgenic organism wherein said transgenic organism is detectable under ultraviolet light.

Another object of the present invention is to provide a transformation system that includes a vector containing a modified *piggyBac* sequence, a sequence for marker expression linked to a polyubiquitin promoter and a nuclear localizing sequence and a helper vector including a heat shock protein gene wherein said system causes an increase in transformation frequency compared to other *piggyBac* transformation systems.

A still further object of the present invention is to provide a vector containing a modified *piggyBac* sequence and an enhanced green fluorescent protein sequence linked to a polyubiquitin promoter and a nuclear localizing sequence.

A still further object of the present invention is to provide a vector that is useful in transforming eukaryotic cells having the sequence SEQ ID No 6.

Another object of the present invention is to provide a transgenic organism that is detectable under ultraviolet light.

A further object of the present invention is to provide a eukaryotic transgenic organism that has been transformed using a transformation system that includes vector containing a modified *piggyBac* sequence, an enhanced green fluorescent protein gene linked to a polyubiquitin promoter and a nuclear localizing sequence, and a helper vector containing a heat shock protein gene promoter.

A still further object of the present invention is to provide a transgenic insect that has been transformed using a vector having the sequence SEQ ID NO 6.

Further objects and advantages of the present invention will become apparent from the following description.

#### Brief Description of the Drawings

Figure 1(a) is a photograph of eye color phenotypes of *Dm*[pBw] transformants.

Figure 1(b) is a photograph of a *w*[m] host strain fly (top) and orange-eye *Dm*[pBw, *gfp*] transformant fly (bottom) under brightfield (left) and UV light (right).

Figure 1 (c) is a photograph of a *w*[m] host strain fly (top) and white-eye *Dm*[pBw, *gfp*] transformant fly (bottom) under brightfield (left) and UV light (right).

Figure 2 (a) is a schematic (not to scale) of the pB[Dmw] vector showing the *Bgl*III, *Sal*I, and *Nsi*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded gray, and the mini-white marker gene is white.

Figure 2 (b) shows an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pBw] transformant sublines, and *w*[m] host strain control samples from transformants using the pBASac (experiment I) or phsp-pBac (experiment II) helpers using *Bgl*III digestion and *Sph*/Hpa *piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female)

designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 2(c) shows a Southern DNA hybridization analysis of Dm[pBw] transformant sublines and w[m] host strain control samples from transformants, using the pBASac (experiment I) or phsp-pBac (experiment II) helpers, using *SalI* digestion and *Hpa/Ase* piggyBac as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 2 (d) shows a Southern DNA hybridization analysis of Dm[pBw] transformant sublines and w[m] host strain control samples from transformants, using the pBASac (experiment I) or phsp-pBac (experiment II) helpers, using *NsiI* digestion and *Nsi/Hpa* + *Hpa/Nsi* probes. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 3(a) is a schematic (not to scale) of the pB[Dmw, PUBnlsEGFP] vector showing the *BglIII*, *XhoI*, and *PstI* restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). The *Sph/Hpa* piggyBac as probe contains 0.67 kb of vector sequence (*SphI* to *BglIII*) with *BglIII* to *HpaI* piggyBac sequence deleted from the vector. Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. PiggyBac vector sequences are shaded gray, the mini-white marker gene is white, and the EGFP marker gene is hatched.

Figure 3(b) is an autoradiogram of a Southern DNA hybridization analysis of Dm[pBw, gfp] transformant sublines, and wild type (wt) and w[m] host strain control samples using *BglIII* digestion and *Sph/Hpa* piggyBac as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines with selected G1 transformant progeny of samples.

Figure 3 (c) is an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pBw, *gfp*] transformant sublines, and wild type (wt) and w[m] host strain control samples using *Xho*I digestion and *Hpa*/Ase *piggyBac* fragment as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with selected G1 transformant progeny of samples.

Figure 3 (d) is an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pBw, *gfp*] transformant sublines, and wild type (wt) and w[m] host strain control samples using *Pst*I digestion and *Hpa*/Ase *piggyBac* fragment + EGFP DNA as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with specific G1 line numbers are given below, with the designation (+) for those expressing visible eye pigmentation and (-) for those having non-pigmented white eyes.

Figures 4 (a) and (b) show inverse PCR strategy to isolate the pB[Dmw] vector insertion site in transformant sublines. Figure 4 (a) is a schematic (not to scale) of the vector insertion in the host plasmid showing the approximate location of the restriction sites and primers used for PCR. Forward (F) and reverse (R) primers are numbered according to their nucleotide position in *piggyBac*. The *piggyBac* sequence is shown in gray surrounded by the TTAA (SEQ ID NO 1) duplicated insertion site, the mini-white marker gene is white, and chromosomal sequence is hatched.

Figure 4(b) shows the *piggyBac* insertion site sequence in p3E1.2 (SEQ ID NOs 7 and 8), and the proximal insertion site sequences (SEQ ID NOs 9 and 10, 11 and 12, and 13 and 14) for three of the transformant sublines.

Figure 5 shows a circular map of the vector pB[PUB-nls-EGFP] #257.

Figures 6a-6f show SEQ ID NO 6 for pB[PUB-nls-EGFP] #257.

Figure 7(a) is a photomicrograph showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUB-nls-EGFP at embryo stages. Under UV light, transformants exhibit bright green

fluorescence, with wild-type non-transformants exhibiting muted yellow autofluorescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 7(b) and 7(c) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUB-nls-EGFP at larval stages. 7(b) is a wild-type non-transformant and 7c is a transformant. Under UV light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluorescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 7(d) and 7(e) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUB-nls-EGFP at adult stages. 7(d) is a wild-type non-transformant and 7(e) is a transformant. Under UV light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluorescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 8(a)-8(e) are eye color phenotypes of *Bactrocera dorsalis* wild-type (+) and white eye (WE) host strain and the Bd[pBCcw] transformant lines 61,115, and 137.

Figures 9(a) and 9(b) show medfly, *Ceratitus capitata* transformed with *piggyBac*/white/EGFP vector (pB[Ccw,pUB-nls-EGFP]) expressing eye color under brightfield (9a) and GFP expression under ultraviolet (9b).

Figure 10 shows a transgenic insect having three integrations observed under ultraviolet light after various times after decapitation. Flies were decapitated at day 0, taped in a plastic box placed outdoors in partial sunlight. Digital photographs were taken each day at the same exposure and magnification.

#### Detailed Description of the Invention

The present invention is an effective transformation system for producing transgenic organisms, especially transgenic insects. The identification and isolation of an autonomous *piggyBac* transposon enables transformation of cells and the production of transgenic organisms wherein DNA capable of being expressed in the



transformed cell or transgenic organism is excised from a transformation construct and inserted into the genome of a cell used to produce a transgenic organism (United States Patent Application 08/844,274; herein incorporated by reference). The term cell for the purposes of this invention includes any cell capable of being transformed by the transformation construct of the present invention and preferably includes any eukaryotic cell.

The term organism for the purposes of the present invention includes any unicellular or multicellular living entity capable of being transformed by the transformation construct of the present invention and preferably includes multicellular eukaryotes. More preferably, the cell or organism is an insect cell or an insect.

The present invention utilizes the transposon machinery of the TTAA (SEQ ID NO 1) specific transposons to excise and insert a targeted functional heterologous DNA sequence into the genome of the host cell. The resulting transformed cell or group of cells are stable transformants that are then used to make a transgenic organism, using techniques known to the skilled artisan, that will pass the introduced gene to all subsequent progeny. The targeted functional heterologous DNA for purposes of this invention is any heterologous DNA capable of being expressed in a host cell and/or a transgenic organism.

The transformation system of the present invention includes a vector, such as, for example, pB[PUB-nls-EGFP] (Figures 5 and 6), that includes a modified piggyBac transposon (pB), a marker construct that includes the enhanced green fluorescent protein gene (EGFP) linked to the promoter region of the *Drosophila melanogaster* polyubiquitin (PUB) gene and the nuclear localizing sequence (nls) of the SV40 virus. This vector can be used to transform and detect transgenic organisms based on expression of the green fluorescent protein marker under ultraviolet light. After chromosomal integration and inheritance of the vector, expression of green fluorescent protein occurs in all cell types, is intense, strongly localized to nuclei, and continues to be detectable under ultraviolet light even after death of the organism. The novel features of this vector includes its construction that deletes about 748 bp of internal piggyBac

sequence without diminishing its function, and the function of the polyubiquitin promoter in a nondrosophilid species. This has utility as a broadly based method for the creation and selection of transgenic organisms, and as a genetic marker for detecting and tracking transgenic insects used in field release programs (Figure 5).

GFP expressivity is critical for nondrosophilid species not amenable to mutant-rescue, it also widens the possibility for using the dominant expression of GFP as a primary transformant marker in many *Drosophila* lines not already carrying the white or rosy mutations, or for screens requiring selection in early development. Though vectors carrying white and *gfp* have been tested previously, the transformations used only white as the transformant selection, with GFP assessed secondarily for specific spatial or developmental expression (Davis et al., Devel. Biol., Volume 170, 726-729, 1995; Wang & Hazelrigg, Nature, Volume 369, 400-403, 1994).

The transformation system of the present invention also includes a piggyBac transposase helper plasmid, pBASac, having its 5' terminus deleted as described by Handler et al. (1998, *supra*; herein incorporated by reference). A new transposase helper under heat-shock promoter regulation was created by the isolation of the 457 bp XbaI-XmnI 5' nontranslated sequence from the *hsp70* gene (Lis et al., Cell, Volume 35, 403-410, 1983, herein incorporated by reference). The heat-shock regulated helper increases the transformation frequency by eight-fold in *Drosophila*, indicating that the piggyBac system could be as effective as routinely used systems such as *P* and *hobo* that have been thus far inactive in nondrosophilids (O'Brochta & Atkinson, Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996).

The creation of a transformed cell requires that the vector containing the functional heterologous DNA first be physically placed within the host cell. Current transformation procedures utilize a variety of techniques to introduce DNA into a cell. In one form of transformation for vertebrate systems, the DNA is microinjected directly into embryos through the use of

micropipettes. Alternatively, high velocity biolistics can be used to propel small DNA associated particles into the cell. In another form, the cell is permeablized by the presence of polyethylene glycol, thus allowing DNA to enter the cell through diffusion. DNA can also be introduced into a cell by fusing protoplasts with other entities that contain DNA. These entities include minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Electroporation is also an accepted method for introducing DNA into a cell. In this technique, cells are subject to electrical impulses of high field strength that reversibly permeabilizes biomembranes, allowing the entry of exogenous DNA sequences. One preferred method of introducing the transformation system of the present invention into insect embryos, in accordance with the present invention, is to microinject fertilized eggs with the vectors of the present invention. The DNA sequence flanked by the transposon inverted repeats will be inserted into the genome of some of the germ cells of the fertilized egg during development of the organism. This DNA will then be passed on to all of the progeny cells to produce transgenic organisms. The microinjection of eggs to produce transgenic animals has been previously described and utilized to produce transformed mammals and insects (Rubin et al., *Science*, Volume 218, 384-393, 1982; Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1986; Morgan et al., *Annu. Rev. Biochem.*, Volume 62, 191-217, 1993; Spradling, A.C., In: *Drosophila: A Practical Approach*, ed. D.B. Roberts, Oxford: IRL Press, 175-197, 1986; all herein incorporated by reference).

Accordingly, a method of producing stably transformed insects includes the step of microinjecting the transformation constructs of the present invention comprising the inverted repeats of a TTAA specific transposon and a helper construct into a cell, preferably a fertile insect egg. This is followed by incubation in an oxygenated and humidified tissue culture chamber at about 22-23° C for about 3-6 hours. Injected cells or eggs are then heat shocked at about 37°-41° C, about 39° C preferred, for about 1 hour. The resulting transformed cells or transgenic organisms have exogenous DNA inserted into the genomic DNA at the sequence TTAA.

Transformed cells and/or transgenic organisms can be selected

from untransformed cells and/or non-transgenic organisms by ultraviolet light since the transformation system includes an enhanced green fluorescent protein gene that produces an altered visible phenotype under ultraviolet light. Using standard techniques known to those familiar with the field, techniques such as, for example, Southern blotting and polymerase chain reaction, DNA can be isolated from transformed cells and/or transgenic insects to confirm that the introduced DNA has been inserted.

Genetic modification of insects with new genetic elements provides a means to control populations of agriculturally pestiferous or beneficial insects. The ability to control pest insects through genetically based sterile insect programs or genetically introduced targeted conditional susceptibilities will result in significant cost savings to agribusiness. This technology can also be used for detection and monitoring of insect populations and infestations where *piggyBac* transgenic insects are present in the population. In addition, introduction of genes that impart resistance to chemicals (including herbicides, pesticides, and insecticides) can improve the efficacy of beneficial insects. Each of these applications will result in more efficient pest control programs.

Enhancing the resistance of beneficial insects to pesticides will enhance the efficacy of the beneficial insects and may allow for the simultaneous use of chemical control and biological control of pests. Some of the beneficial insects that would make good candidates for such transformations include Hymenopteran parasitoids of *Heliothis* spp.: *Microplitis croceipes* and *Cardiochiles nigriceps*; Hymenopteran parasitoid of Diamondback moth, *Plutella xylostella*: *Diadegma insolare*; Hymenopteran parasitoid of the Indianmeal moth, *Plodia interpunctella*: *Bracon hebitor*; and Hemipteran predators: *Xylocoris flavipes*, *Podisus maculatus*.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention as described by the claims. *Drosophila melanogaster* white strain w[m], was used in the following examples

as a model system for transformation system studies using the vectors of the present invention. *D. melanogaster* and transformant progeny were maintained at about 23-25° C on standard cornmeal-yeast-molasses media.

#### EXAMPLE 1

The *piggyBac* transposase helper plasmid, pBASac, having its 5' terminus deleted was described previously (Handler et al., 1998; *supra*, herein incorporated by reference). pBASac was created by digestion of p3E1.2 (United States Patent Application 08/844,274) with *SacI* and religation, that deletes the 5' *piggyBac* terminal sequences but maintains the putative *piggyBac* promoter region. A transposase helper under heat-shock promoter regulation was created by isolation of the 457 bp *XbaI-XmnI* 5' nontranslated sequence from the *hsp70* gene (Lis et al., 1983, *supra*; herein incorporated by reference). The *XbaI-XmnI* fragment was blunted and ligated into the *SacI*-blunted site of pBASac to create phsp-pBac. This places the *hsp70* promoter sequence upstream of the putative *piggyBac* promoter.

The pB[Dmw] vector was created by insertion of a *Drosophila melanogaster* mini-white gene (Pirrotta et al., EMBO J., Volume 4, 3501-3508, 1985; herein incorporated by reference) into the 3E1 *piggyBac* element within the 6.0 kb p3E1.2 plasmid (Cary et al., 1989, *supra*). The mini-white gene was isolated as a 4.2 kb *EcoRI* fragment, blunted and ligated into the p3E1.2 *HpaI* site. The inserted *w* gene interrupts the *piggyBac* open reading frame (ORF), but otherwise leaves the *piggyBac* element intact, with the respective promoters in opposite orientation. A *piggyBac* vector marked with *w* and *gfp* was created by initial construction of *piggyBac* marked with an enhanced *gfp* regulated by *D. melanogaster* polyubiquitin (PUB) promoter (Lee et al., Mol. Cell. Biol., Volume 8, 4727-4735, 1988; herein incorporated by reference) linked in-frame to the SV40 nuclear localizing sequence (nls) (Lanford et al., Mol. Cell. Biol., Volume 8, 2722-2729, 1986). The

polyubiquitin-nls (PUB-nls) cassette from PUBnlsGFP (Davis et al., 1995, *supra*) was isolated as *KpnI-SmaI* fragment and inserted into the *KpnI-SmaI* cloning site of EGFP-1 (Clontech) (Cormack et al., *Gene*, Volume 173, 33-38, 1996; Yang et al., *Nucleic Acid Res.*, Volume 24, 4592-4593, 1996). Polyubiquitin-nls-EGFP was then isolated as a 4.1 kb *BglIII-StuI* fragment and ligated into the *BglIII-HpaI* site of *piggyBac* within p3E1.2 to create pB[PUBnlsEGFP]. The *BglIII-HpaI* digestion results in a 748 bp deletion within p3E1.2. The mini-white gene was then inserted into the unique *BglIII* site by blunt-end cloning to create pB[Dmw, PUBnlsEGFP].

#### EXAMPLE 2

Embryo injections used standard procedures (Rubin & Spradling, *Science*, Volume 218, 348-353, 1982; herein incorporated by reference) with dechoriation achieved either manually or by 1.6% hypochlorite solution followed by about 2 washings in approximately 0.02% Triton-X 100 in water. Eggs were placed on double-stick tape, desiccated in room-air for about 10-15 minutes and submerged under Halocarbon 700 oil. Injections followed standard *Drosophila* microinjection procedures (Rubin and Spradling, *Science*, Volume 218, 348-353, 1982; herein incorporated by reference). DNA mixtures had vector:helper concentrations of about 600:400  $\mu\text{g/ml}$ , respectively, in injection buffer (approximately 5 mM KCl; approximately 0.1mM sodium phosphate; at about pH 6.8). Injected eggs were placed in an oxygenated and humidified tissue culture chamber at about 22-23°C for about 3-6 hours, and *phsp-pBac* injected eggs were heat shocked at about 37°C for about one hour. Hatched larvae were collected about 1-2 days later and placed on larval diet. Eclosed G0 male adults were mated either individually to about 2 or 3 w[m] virgin female adults, or in groups of about three females to about six males. G1 eggs were collected for two weeks and reared under standard conditions that include maintaining the eggs at about 23-25° C on standard cornmeal-yeast-molasses media (Ashburner et al., *supra*).

Green fluorescent protein (GFP) was observed at all

developmental stages under a Leica MZ-12 stereozoom microscope using a mercury lamp and a ultraviolet longpass filter set (HQ 41012 FITC; Chroma) optimized for red-shifted GFP variants. Photographic documentation used an Olympus OM-4 camera and 400 ASA Fujichrome film with exposure times that were determined empirically.

In the first of three transformation experiments, the *piggyBac* vector system was tested in *D. melanogaster* white strain using a helper transposase under *piggyBac* regulation (pBASac) and a vector marked solely with *D. melanogaster* mini-white gene (pB[Dmw]). A mixture of vector and helper plasmids at concentrations of about 600 and about 400 µg/ml, respectively, was injected into about 2,650 embryos from that about 418 larvae hatched with about 283 emerging as adults. (See Table 1 below). The G0 adults were backcrossed to w[m] flies in groups totaling about 111. Four of the G0 lines yielded G1 offspring having varying levels of eye pigmentation (Figure 1). One line (F30) was sterile, and one line produced only white eye offspring, and therefore only two of the putative Dm[pBw] transformants were verified. One of these (F13) exhibited eye pigmentation only in females in several succeeding generations, suggesting that the integration caused a sex-linked lethal mutation. Presuming a fertility rate of about 50% (fertility rates are typically between about 40-60%; see below), an approximate transformation frequency of about 1-3% of fertile G0s was obtained.

In a second experiment, the pB[Dmw] vector was again tested but with a *piggyBac* transposase helper under *D. melanogaster hsp70* (Lis et al., 1983 *supra*) promoter regulation (phsp-pBac). A vector/helper mixture, at a concentration of approximately 600/400 µg/ml was injected into about 1,940 embryos, of which about 247 larvae hatched, with about 122 emerging as adults (See Table 1, below). G0 adults were initially backcrossed in a total of about 49 groups to w[m] flies, after which they were individually mated to determine fertility. Of the about 98 surviving G0 flies, about 41 yielded offspring resulting in a fertility rate of about 42%. Of the 41 fertile G0 flies, 11 lines produced offspring having

varying levels of eye coloration (Figure 1) yielding a transformation frequency of about 26%. The number of G1 offspring from the G0 lines varied considerably, ranging from 1 G1 in lines M11 and F1, to 102 G1 flies in line M13.

In a third experiment, the phsp-pBac helper was used, but with a piggyBac vector including the enhanced green fluorescent protein (gfp) marker gene in addition to the *D. melanogaster* white gene. This allowed the testing of a new gfp marker construct in transformants that could be primarily identified by white expression. Although expression of wild type GFP under polyubiquitin-nuclear localizing sequence regulation had been tested previously in *D. melanogaster* P transformants (Davis et al., 1995, *supra*), the vector of the present invention improves expression of GFP by using an enhanced GFP (EGFP-1) having a double mutation causing a reported increase in expression of up to about 35-fold (Cormack et al., 1996, *supra*; Yang et al., 1996, *supra*). The variant form is also optimized for mammalian codon usage and polyadenylation, and preliminary tests of the marker construct indicated transient GFP expression in both *Drosophila* embryos and dipteran and lepidopteran cell lines (A.M. Handler and R.A. Harrell, unpublished). The vector construct, pB[Dmw, PUBnlEGFP], also allowed evaluation of piggyBac transformation with about a 10.0 kb vector, approximately 3.4 kb larger than previous vectors tested, and having about 748 bp of piggyBac DNA deleted (previous vectors retained all piggyBac DNA). As before, a mixture of about 600 µg/ml vector and about 400 µg/ml helper was injected into about 2147 embryos, of which about 412 larvae hatched, and about 218 emerged as adults (Table 1 below). G0 adults were backcrossed to w[m] flies in a total of about 90 mating groups, of which about 79 yielded offspring. Although white gene expression (eye pigmentation) was depended upon as the primary marker, G1 larvae and pupae were examined under UV for visible GFP expression, and seven of the G0 lines yielded fluorescent G1 larvae and pupae. Interestingly, as shown below in Table 2, upon adult emergence only six of the seven G0 lines



yielded G1 offspring with observable eye color pigmentation. While about 70 G1 offspring in total exhibited observable green fluorescence, only about 27 of these flies exhibited a level of eye pigmentation that would have allowed their selection under normal screening procedures. In contrast, all of the G1 flies with eye color pigmentation expressed GFP. Figure 1b shows a Dm[pBw, egfp] transformant having an orange eye color and GFP fluorescence, with no fluorescence observed in the w[m] host. Figure 1c shows another transformant having a white eye phenotype indistinguishable from that in the w[m] host strain, but exhibiting an equal, if not greater level of GFP fluorescence compared to the orange eye transformant. Notably, fluorescence is quenched in the eye of the pigmented transformant, while it is easily visible in the white eye transformant. High magnification examination revealed a few pigmented ommatidia in some white eye G1 flies expressing GFP, though these would not have been normally detected. Based on selection by GFP expression and presuming about 50% fertility, an approximate transformation frequency of about 6-7% of fertile G0 flies is deduced.

An assessment of vector activity based on germline transformation frequency is a factor of both transposon mobility in the host embryo and levels of genomic position effect suppression of the marker gene, or stated more simply, the ability to visibly identify putative transformants. While position effect variegation and suppression of white expression in transformants is well established (Hazelrigg et al., Cell, Volume 64, 1083-1092, 1984; Pirodda et al., 1985, *supra*), the effect of complete marker suppression on transformation frequencies has not been assessed since such transformants have been only detected fortuitously after molecular analysis. The experiment using both the white and GFP markers proved the importance of position effects on marker expression convincingly, since GFP was readily detectable in 70 G1 flies, yet eye pigmentation was apparent in less than 40% of these. Under typical screening procedures these flies would not have been scored as transformants, though pigmentation in a few ommatidia in some flies could be detected at high magnification, and for a few lines, pigmentation was more apparent in subsequent

generations. It is likely that expression of the *white* marker would have been improved by heat shock regulation, but nonetheless, GFP was easily detected in all the non-pigmented transformants, and strongly expressed in some. The influence of modifier genes on position effect variegation is complex, and target genes (or their promoters) are not equivalently affected (Bhadra et al., *Genetics*, Volume 150, 251-263, 1998). The polyubiquitin-*gfp* gene may be a target of position effect modifiers, but it is clearly less susceptible to suppression relative to *white* in terms of its expressed phenotype in the same chromosomal context. The data suggests that GFP is a more reliable visible marker than *white*, that portends well for its use as a general marker in other insects.

TABLE 1. Transformation Experiments.

Expt	vector/ helper	eggs injected	G0s mated	% fertility	No. G0 lines	No. G1 lines	transformant frequency
I	pB[Dmw]/pBASac	2,650	283	nd	4	11	0.01-0.03*
II	pB[Dmw]/phsp-pBac	1,940	122	42	11	266	0.26
III	pB[Dmw, PUbnsEGFP]/phsp- pBac	2,147	218	nd	7	70	0.06-0.07*

\*estimated frequency based on 50% fertility

TABLE 2. G1 white<sup>+</sup> and GPR marker expression in Dm[pBw, gfp] transformants.

G0 line	No. G1	GFP	white <sup>+</sup>	Frequency white <sup>+</sup>
M4	4	4	3	0.75
M9	21	21	2	0.10
M12	3	3	1	0.33
M23	15	15	14	0.93
M45	5	5	0	0
M47	21	21	6	0.29
F10	1	1	1	1.00
Total	70	70	27	0.39

### EXAMPLE III

Southern hybridization was performed to verify genomic transposition of the *piggyBac* vectors. Approximately 5-10 µg of genomic DNA was digested with indicated restriction enzymes and separated on about 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were labeled with [<sup>32</sup>P]-dCTP by random priming (Gibco BRL) according to the manufacturer's specifications. Probe DNA was generated from indicated *piggyBac* restriction fragments (see below) that were separated from p3E1.2, or the entire *egfp* gene from pEGFP-1 (Clontech) by agarose electrophoresis and gel-elution. Hybridizations were performed in phosphate buffer, approximately pH 7.5; about 1% BSA; about 7% SDS at about 65°C with an initial wash in about 2X SSC; about 0.2% SDS at about room temperature and about two washes in about 1X SSC; about 0.1% SDS at about 55°C for approximately 30 minutes. Autoradiography was performed by exposure of Kodak X-Omat film at about -90° C.

Genomic transposition of the *piggyBac* vectors was verified by Southern DNA hybridization. The basic strategy was to perform hybridizations to the 5' vector arm using the *piggyBac* *Sph*I-*Hpa*I or *Nsi*I-*Hpa*I fragment as probe, and the 3' vector arm using the *Hpa*I-*Ase*I or *Hpa*I-*Nsi*I fragment as probe. Using probes to both

vector arms, internal fragments spanning most of the vector were detected. Hybridizations to the vector arms and adjacent chromosomal sequence indicate their presence in non-plasmid DNA and indicate the number of integrations, while internal hybridizations that yield known fragment sizes confirm vector integrity.

For pB[Dmw] transformants, genomic DNA was initially digested with *Bgl*III and hybridized to the labeled *Sph*-*Hpa* *piggyBac* fragment, that detects both vector arms resulting in two bands for each integration (Figure 2A). Each intact vector integration should result in one band greater than about 0.67 kb for the 5' arm, and one band greater than about 5.9 kb for the 3' arm. Since varying eye color phenotypes among G1 sublines was observed, and in some cases within G1 sublines, sublines having light orange, dark orange, or red eye coloration from the same G1 sublines were selected for hybridization analysis. For example, flies having differing phenotypes from lines M13-39, M19-90, and M19-91 were hybridized separately, but no difference in the number or sites of insertion were apparent. Of all the lines tested, all had single integrations except for two lines having two integrations (M13-39 and M19-91) and one line having three integrations (F14-63). All the lines with multiple integrations had dark orange or red eye color, though several lines with a single integration also shared these phenotypes. Hybridization patterns for the lines tested indicated that for most of the G0 lines, different integrations were transmitted to many of the G1 sibling offspring. For example, the three G1 sublines tested from both the M3 and M5 G0 lines all show different patterns indicating at least three independent integrations occurring in the two G0 germlines.

Genomic DNA digested with *Sal*I and hybridized to *Hpa*I-*Ase*I probe yielded single bands greater than about 3.0 kb for each integration, and the number of integrations determined were consistent with the *Sph*I-*Hpa*I hybridizations (Figure 2B). For all samples, *Nsi*I digestion and hybridization to *Nsi*-*Hpa*I and *Hpa*I-*Nsi*I probe yielded only about 1.5 kb and about 4.6 kb bands accounting for about 6.1 kb of the about 6.6 kb vector, indicating the same generally high level of vector integrity for all

integrations tested.

G1 sublines from six G0 lines transformed with the pB[Dmw, PUbnsEGFP] vector were digested with either *Bgl*II and probed with *Sph*I-*Hpa*I piggyBac DNA for 5' vector arm analysis, or digested with *Xho*I and probed with *Hpa*I-*Ase*I piggyBac DNA for 3' arm analysis (Figure 3A and 3B). Both hybridizations yielded one band for each sample, indicating single integrations having occurred in each line. *Nsi*II restriction digests with *Nsi*II-*Hpa*I and *Hpa*I-*Nsi*II hybridizations yielded about 0.7 kb and about 0.8 kb bands indicating vector integrity for each integration (data not shown).

Two G0 lines, M9 and M47, yielded a high proportion of G1 flies expressing only GFP and white eyes, and line M45 that yielded only white eye transformants. These lines were analyzed by *Pst*I digestion and hybridization to EGFP and *Hpa*-*Ase*. All lines shared the about 4.4 kb internal vector fragment, with an additional junction fragment from the 3' vector arm and adjacent insertion site chromosomal DNA. The M9 white eye lines all shared the same integration indicated by an about 0.9 kb junction fragment, and similarly the M47 white eye lines all shared the same 5.0 kb junction fragment. The pigmented lines M9-2 and M9-3 had different integrations from each other, and from their white eye sibling lines, and the pigmented lines M47-9 and M47-10 shared the same integration based on an about 4.0 kb junction fragment, but which differs from their white eye siblings. These hybridizations, and that for M45-1, proves that the white eye flies were transformed, and that white expression was likely influenced by differing insertion sites from their pigmented sibling lines.

#### EXAMPLE IV

To verify that piggyBac-mediated chromosomal transpositions had occurred, insertion sites were isolated by inverse PCR from sublines F1-2, M17-4 and M31-6, all having single integrations. Inverse PCR was performed as described previously (Handler et al., 1998, *supra*; herein incorporated by reference) using *Hae*III digestions for 5' and 3' junctions and *Msp*I digestion for 3'

junctions. After about 4 hours digestion, restriction fragments were circularized by ligation at about 16° C for about 16 hours. PCR was preformed on the circularized fragments by using primer sequences in opposite orientation within the *piggyBac* restriction site and terminus for each junction. For the 5' junction, the forward primer (572F) 5'-TCTTGACCTTGCCACAGAGG-3' (SEQ ID NO 2) and reverse primer (154R) 5'-TGACACTTACCGCATTGACA-3' (SEQ ID NO 3) were used. For the 3' junction the reverse primer (2118R) 5'-GTCAGTCCAGAAACAACCTTTGGC-3' (SEQ ID NO 4) and the forward primer (2385F) 5'-CCTCGATATACAGACCGATAAAAAACACATG-3' (SEQ ID NO 5) were used. PCR products were separated in low-melting-temperature agarose, and fragments were selected that were longer than the respective restriction site terminus distances and different from those expected from the p3E1.2 based vector and helper plasmids. These products were directly subcloned into ddT vectors (Invitrogen), that were sequenced by using primers to vector sequence proximal to the respective termini. Subcloned PCR products were sequenced and analyzed by alignment using GeneWorks 2.5 software (Oxford Molecular Group) and subjected to BLAST analysis (Altshul et al., J. Mol. Biol., Volume 215, 403-410, 1990; herein incorporated by reference) to identify genomic insertion site sequences and distinguish them from those in the injected plasmids. For all the integrations both the 5' and 3' junctions yielded the *piggyBac* inverted terminal repeat sequences immediately adjacent to a TTAA sequence (SEQ ID NO 1) and proximal insertion site DNA (Figure 4). The TTAA (SEQ ID NO 1) duplicated target site is characteristic of all *piggyBac* integrations (Elick et al., Genetica, Volume 97, 127-139, 1995) and typically indicates a vector-mediated transposition. The BLAST analysis revealed that the M17-4 integration occurred in a TTAA site within the *cubitus interruptus*-Dominant gene located on chromosome 4 at nucleotide 12,898 (GenBank submission U66884; Ahmed & Podemski, Gene, Volume 197, 367-373, 1997), and the M3106 integration was found to have occurred in a TTAA site within a previously sequenced region of the distal X chromosome (GenBank submission AL09193; Murphy et al, direct submission). Determination of insertions in these previously sequenced sites gives the first

direct proof that a *piggyBac* vector does indeed insert into and duplicates TTAA (SEQ ID NO 1) insertion sites in a eukaryotic genome.

Two of the three insertion sites that were sequenced were found to be in previously sequenced genomic loci, and as expected, the insertion sites were all TTAA (SEQ ID NO 1) with one of them within the *ci<sup>D</sup>* allele on the fourth chromosome. Many transposons have insertion site preferences, and for at least some, a clear negative bias against specific sites or loci. This has been clearly demonstrated by genomic hotspots and coldspots for *P* integration in *D. melanogaster* (See Engels, In: *Mobile DNA*, D.E. Berg and M.M. Howe, eds., American Society of Microbiology, Washington, D.C., 439-484, 1989), and by differences in preferential integration sites between *hobo* and *P* (Smith et al., *Genetics*, Volume 135, 1063-1076, 1993). If the TTAA (SEQ ID NO 1) specificity for *piggyBac* integration is not further influenced by proximal sequences, then *piggyBac* transpositions may find use in transposon-mutagenesis and enhancer traps for loci refractory to *P* or *hobo* transpositions in *Drosophila*.

#### Example V

The Caribbean fruit fly, *Anastrepha suspensa*, was transformed with a *piggyBac* vector marked solely with PUb-nls-GFP (pB[PUb-nls-EGFP]) (Figures 5 and 6) using the *hsp70-piggyBac* (phsp-pBac) helper. From injected embryos, 561 surviving G0 adults were intermated in 60 small groups. Four of the G0 groups yielded a total of 57 G1 offspring exhibiting green fluorescence at all stages of development (See Figure 7) and chromosomal vector integrations were verified by Southern hybridization for each G0 group. To test GFP as a genetic marker for field released transgenic flies, the perdurance of GFP expression was assayed in transgenic flies killed by decapitation. Two to three day old *A. suspensa* adults transformed with pB[PUb-nls-EGFP], and wild type non-transformed adults, were decapitated and placed within a plastic box kept outdoors in partial shade. GFP fluorescence was

observed daily by digital images taken with a SPOT-1 cooled CCD digital camera (Diagnostic Instruments, Inc.) through a Leica MZ-12 stereozoom microscope. All images were taken at the same magnification and exposure parameters. Figure 10 shows that while GFP fluorescence decreases with time after death, unambiguous detection of GFP is still possible at 28 days after decapitation, with no fluorescence detectable in wild flies. This indicates that the PUB-nls-EGFP marker should be a reliable visible detection system for released transgenic insects, and especially for those captured and killed in field traps with monitoring occurring after extended time periods.

#### Example VI

A piggyBac vector marked with the Mediterranean fruit fly (*Ceratitidis capitata*) white gene cDNA (pB[Ccw]) and the phsp-pBac helper was used to transform the oriental fruit fly (*Bactrocera dorsalis*). Injected G0 embryos from the *B. dorsalis* white eye mutant strain yielded 102 fertile adults, that upon individual backcrossing, yielded three lines of putative transformants with pigmented eyes (Figures 8a-8e). One of these lines produced 119 G1 transformants. Southern DNA hybridization analysis with piggyBac and white gene probe verified chromosomal integration of the piggyBac-white vector in all three lines. In a separate experiment, the white/PUB-nls-EGFP marker within pB[Ccw, PUB-nls-EGFP] was introduced into a single *B. dorsalis* transformant line from 17 G0 matings. As in *Drosophila*, the transformant was selected solely by GFP expression, having undetectable eye coloration. This reaffirms the notion that the polyubiquitin-EGFP marker is significantly more reliable than white gene markers.

#### Example VII

The PUB-nls-EGFP marker was introduced into the medfly, *Ceratitidis capitata*, to further test GFP as a transgenic selection, and to create GFP-marked strains for testing as a field release marker in medfly SIT. First a piggyBac vector marked with PUB-



nls-GFP and the medfly white gene (pB[Ccw, Pub-nls-EGFP]) was tested, and then the vector solely marked with Pub-nls-GFP pB[Pub-nls-EGFP]) was tested. Both experiments used the *hsp70*-piggyBac (phsp-pBAC) helper. Based on GFP fluorescence, the first experiment yielded five transformant lines from 99 fertile G0s (See Figure 9), while the second experiment yielded three transformed lines from 17 fertile G0s. Transformation was verified by Southern hybridization analysis. The foregoing detailed description is for the purpose of illustration. Such detail is solely for that purpose and those skilled in the art can make variations without departing from the spirit and scope of the invention.

We claim:

1. A transformation system comprising a vector containing a nucleotide sequence from a piggyBac transposon in which is inserted an enhanced green fluorescent protein gene linked to a promoter region of a polyubiquitin gene and a nuclear localizing sequence of an SV40 virus.
2. A transformation system of claim 1 further comprising a piggyBac transposase helper plasmid under heat-shock promoter regulation.
3. The transformation system of claim 1 wherein said polyubiquitin gene is from *Drosophila melanogaster*.
4. The transformation system of claim 2 wherein said polyubiquitin gene is from *Drosophila melanogaster*.
5. The transformation system of claim 1 wherein said piggyBac transposon is modified by deleting about 748 bp of internal piggyBac sequence.
6. A vector having SEQ ID NO 6.
7. A transgenic organism transformed using the transformation system of claim 3 wherein in said organism is detectable under ultraviolet light.
8. A transgenic insect transformed using the transformation system of claim 3 wherein said insect is detectable under ultraviolet light.
9. The transgenic insect of claim 8 that continues to be detectable under ultraviolet light after death.

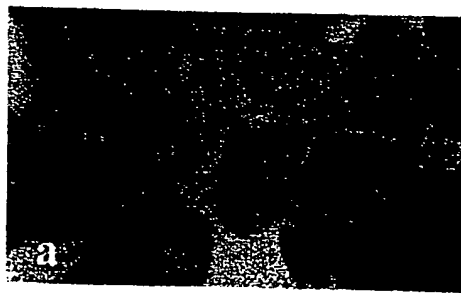


FIG. 1a



FIG. 1b



FIG. 1c

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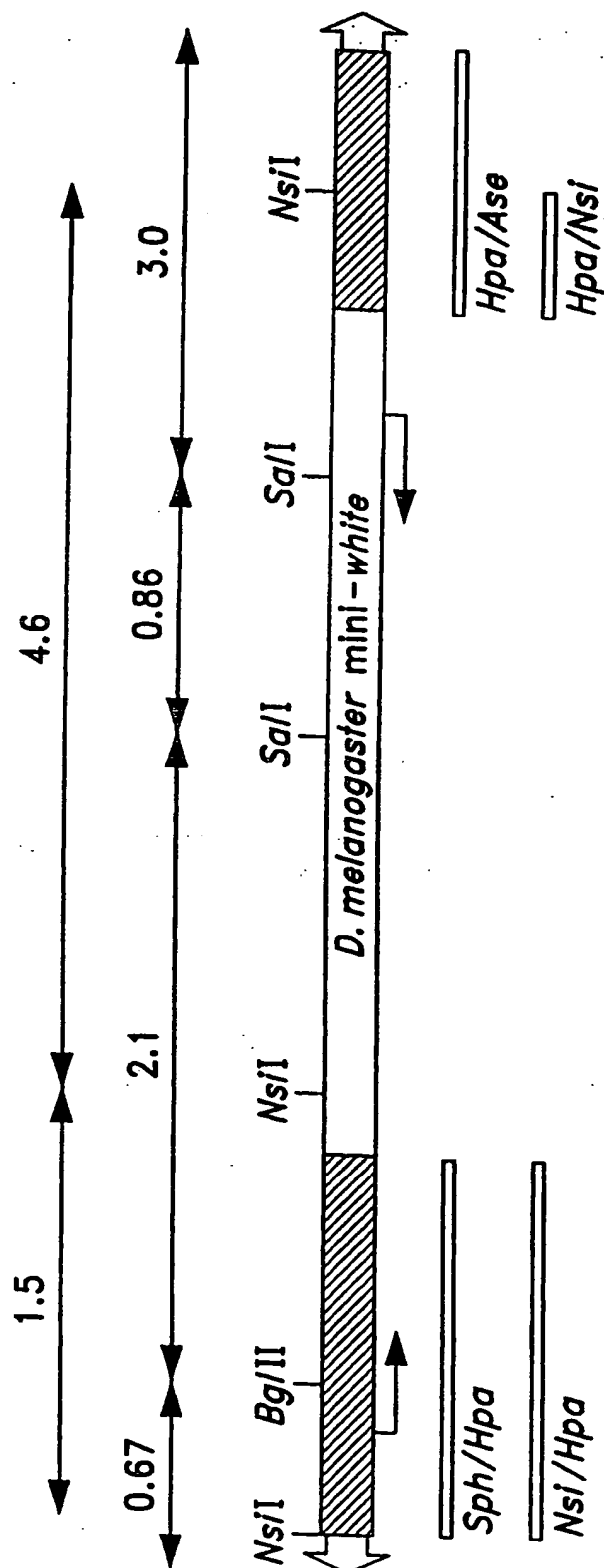


Fig. 2a

*Bgl*III digestion - *Sph*/Hpa probe

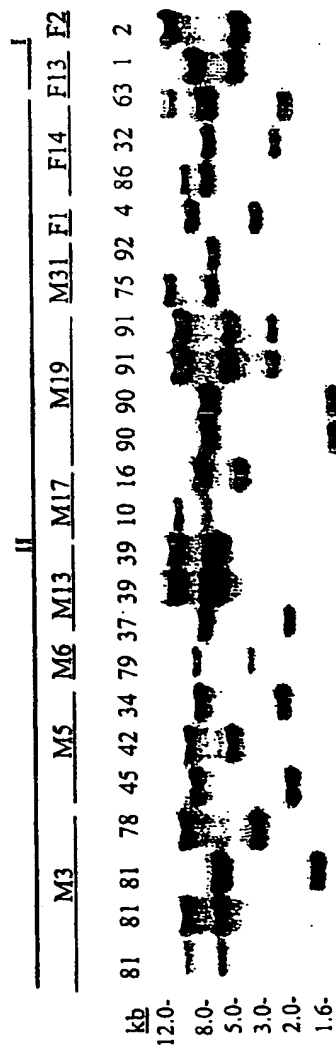


FIG. 2b

*Sa*II digestion - *Hpa*/*Ase* probe

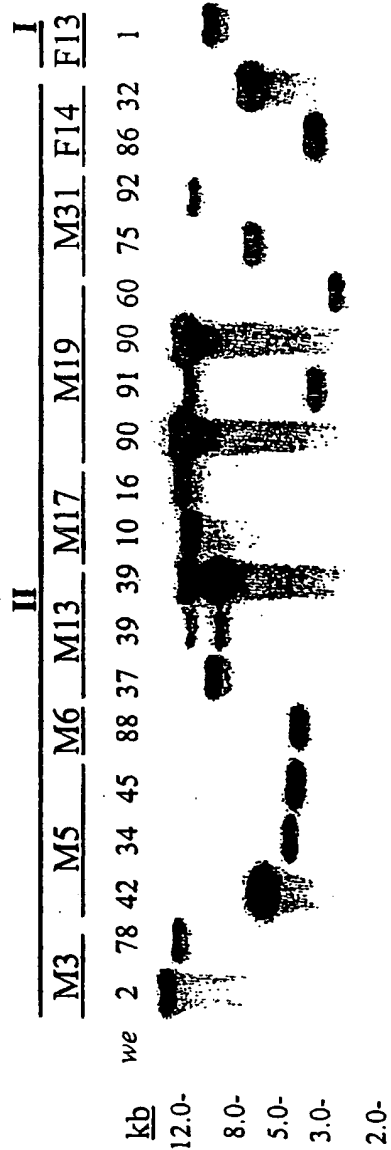


FIG. 2c

*Nsi*I digestion - *Nsi*I/*Hpa*I + *Hpa*I/*Nsi*I probes

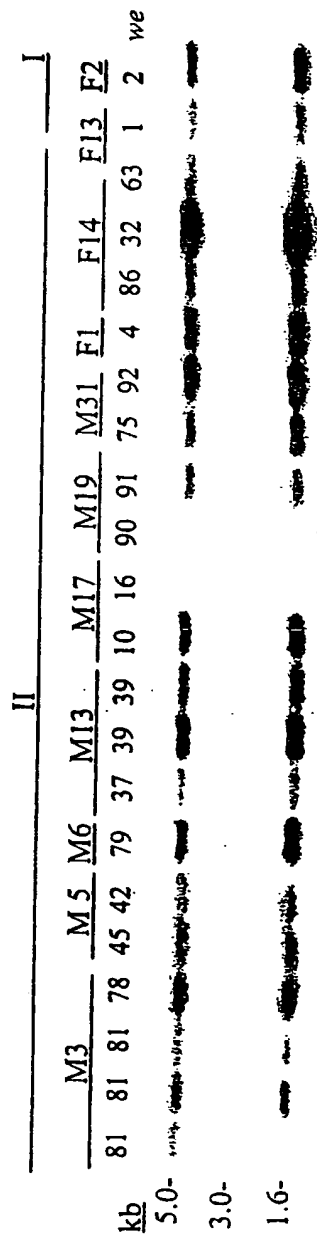
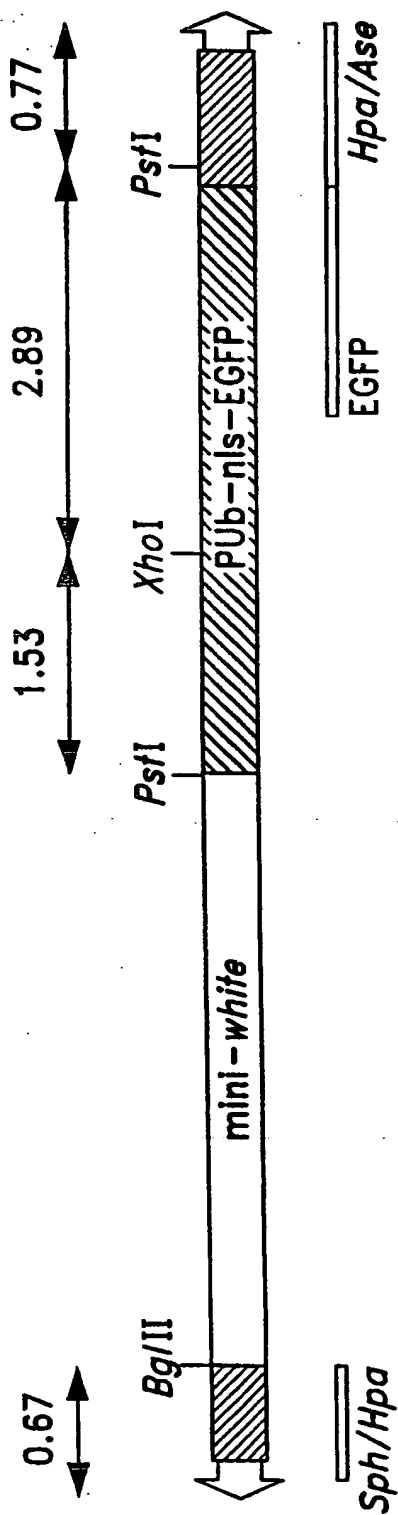


FIG. 2d



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Fig. 3a



*Bgl*II digestion - *Sph*/Hpa probe      *Xho*I digestion - *Hpa*/Ase probe

kb    M9   M4   M12 M23 M47 F10      M9   M4   M12 M23 M47 F10    wt

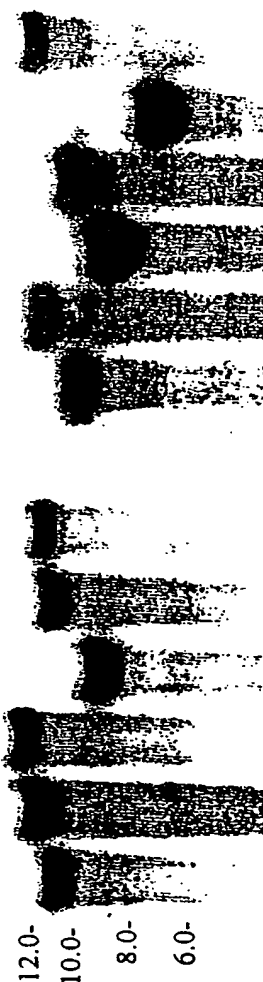


FIG. 3b      FIG. 3c

*Pst*I digestion - *Hpa*/*Ase*+EGFP probe

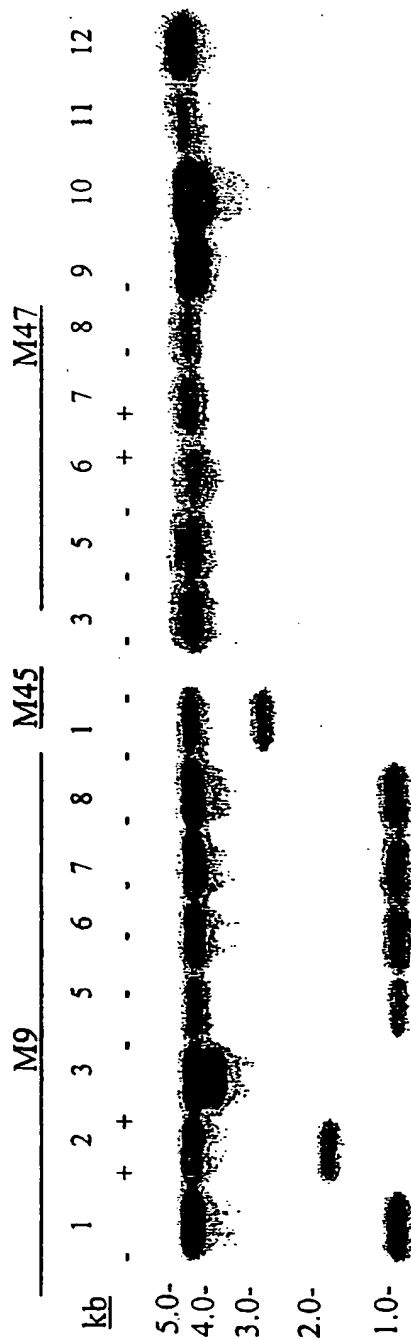


FIG. 3d

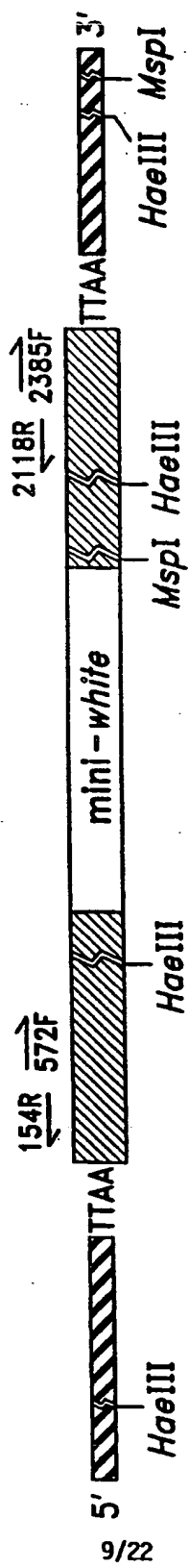


Fig. 4a

p3E1.2 aagcgaaatcttcttTTAA-piggyBac-TTAAataatagttctctaatt

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F1-2 aaaagactgacttatTTAA-piggyBac-TTAAtaagcacactgagtc

M17-4 aaaatgtcgtctaggTTAA-piggyBac-TTAAagccgtatatcagat

M31-6 aaatgaacgactcttcttTTAA-piggyBac-TTAAtggtcttcttagttgt

FIG. 4b

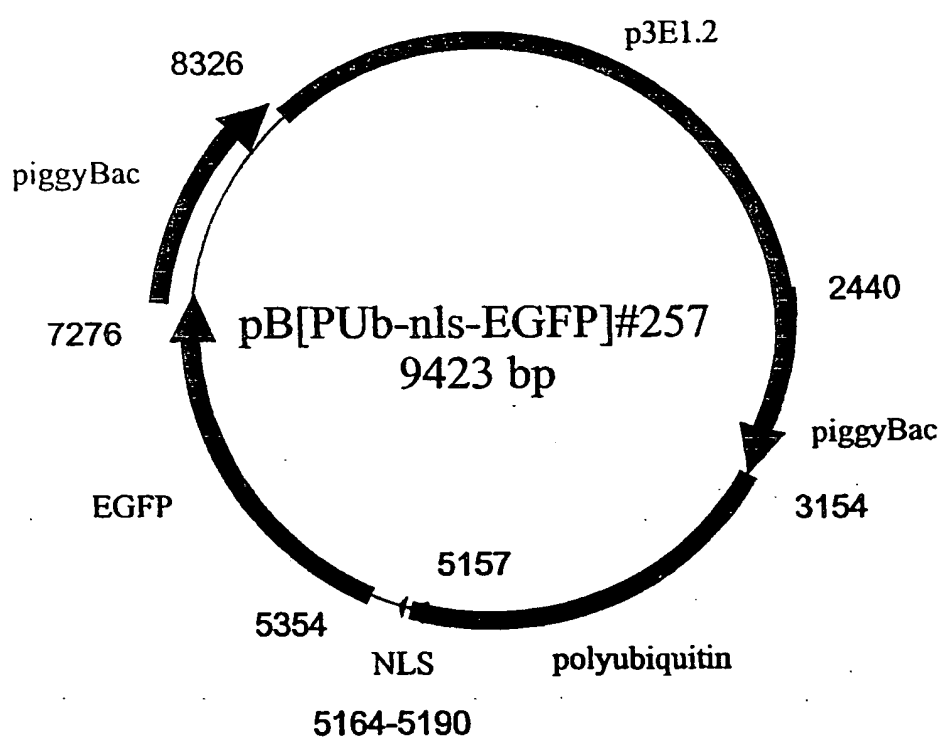


FIG. 5

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GACGAAAGGG	CCTCGTGATA	CGCCATTTTT	TATAGGTTAA	TGICATGATA	50
ATAATGGTTT	CTTAGACGTC	AGGTGGCACT	TTTCGGGGAA	ATGTCGGCGG	100
AACCCCTATT	TGTTTATTTT	TCATAATACA	TTCAAATATG	TATCCGCTCA	150
TGAGACAATA	ACCTTGATAA	ATGCTTCAAT	AATATTGAAA	AAGGAAGAGT	200
ATGAGTATTC	AACATTTCCG	TGTGGCCCTT	ATTCCCTTTT	TTCGGGCATT	250
TTCGCTTCCT	GTTTTTGCTC	AACCAGAAAC	GCTGGTGAAA	GTAAAAGATG	300
CIGAAGATCA	GTTCGGTGCA	CGAGTGGGTT	ACATCGAACT	CGATCTCAAC	350
AGCGGTAAGA	TCCTTGAGAG	TTTTGGCCCC	GAAGAAGGTT	TTCGAATGAT	400
GAGCACTTTT	AAAGTTCIGC	TATGTGGCGC	GGTATTATCC	CGTATTGACG	450
CCGGGCAAGA	GCAACTCGGT	CGCCGCATAC	ACTATCTCA	GAATGACTTG	500
GTGAGTACT	CACCAGTCAC	AGAAAAGCAT	CTTACGGATG	GCATGACAGT	550
AAGAGAATTA	TGCAGTGTCT	CCATAACCAT	GAGTGATAAC	ACTGGGGCCA	600
ACTTACTTCT	GACAACGATC	GGAGGAACGA	AGGAGCTAAC	CGCTTTTTTG	650
CACAACATGG	GGGATCATGT	AACTCGCCTT	GATCGTTGGG	AACCGGAGCT	700
GAATGAAGCC	ATACCAAACG	ACGAGCGTGA	CACCACGATG	CCGTGTAGCA	750
TGGCAACAAC	GTTCGGCAAA	CTATTAACTG	GCGAATCTCT	TACTCTAGCT	800
TCCCGGCAAC	AATTAAATAGA	CTGGATGGAG	GCGGATAAAG	TTCAGGACC	850
ACTTCTGCGC	TCCGGCCCTC	CGGCTGGCTG	GTTTATTGCT	GATAAATCTG	900
GAGCCGGTGA	GCGTGGGICT	CGCGGTATCA	TTCGAGCACT	GGGGCCAGAT	950
GGTAAGCCCT	CCCGTATCGT	AGTTATCTAC	ACGACGGGGA	GTCAGGCAAC	1000
TATGGATGAA	CGAANTAGAC	AGATCGCTGA	GATAGGTGCC	TCACTGATTA	1050
AGCATTTGGTA	ACTGTCAGAC	CAAGTTTACT	CATATATACT	TTAGATTGAT	1100
TTAAAACTTC	ATTTTTTAATT	TAAAAGGATC	TAGGTGAAGA	TCCTTTTIGA	1150
TAATCTCATG	ACCAAAATCC	CTTAACGTGA	GTTTTTGTTC	CACTGAGCGT	1200
CAGACCCCGT	AGAAAAGATC	AAAGGATCCT	CTTGAGATCC	TTTTTTTCTG	1250
CGCGTAACTT	GCCTGCTTGA	AACAAAAAAA	CCACCGCTAC	CAGCCGGTGT	1300
TGTGTTTGGG	GATCAAGAGC	TACCAACTCT	TTTTTCGAAG	GTAACCTGGT	1350
TCAGCAGAGC	GCAGATACCA	AATACGTGTC	TTCTAGTGTA	GCCGTAGTTA	1400
GGCCACCACCT	TCAAGAACCT	TGTAGCAACG	OCTACATACC	TGGCTCTGCT	1450
AATCCGTGTA	CCAGTGGCTG	CTGCCAGTGG	CGATAAGTGG	TGCTTTACCG	1500
GGTTGGACTC	AAGACGATAG	TTACCGGATA	AGGCGCAGCG	GTCGGGCTGA	1550
ACGGGGGGTT	CGTGCACACA	GCCAGCTTGG	GAGCGAAGCA	CCATACACGA	1600
ACTGAGATAC	CTACAGCGTG	AGCATTGAGA	AAGCGCCACG	CTCCCGAAG	1650
GGAGAAAGGC	GGACAGGTAT	CCGGTAAAGC	GCAGGGTCCG	AACAGGAGAG	1700
CGCACGAGGG	AGCTTCCAGG	GGGAAAGGCC	TGGTATCTTT	ATAGTCTCTG	1750
CGGGTTTGGC	CACCTCTGAC	TTCAGCGTGG	ATTTTGTGTA	TGCTGTCTAG	1800

FIG. 6a

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GGGGGGGGAG	CCATATGGAA	AACGCCAGCA	ACGGGGCCCT	TTTACGGTTC	1850
CTGGGCTTTT	GCIGGGCTTT	TGCTACATG	TCTTTTCIG	CGTTATCCCC	1900
TGATTCTGIG	GATAACGGTA	TTACGGCTTT	TGAGTACGCT	GATAACGGCTC	1950
GGGGCAGCCG	AACGACCGAG	CGCAGCGAGT	CAGTACGCGA	GGAAGCGGAA	2000
GAGGGCCCAA	TACGCAAACC	GCTCTCCCC	GGGGTTCGGC	CGATTCAATTA	2050
ATGCAGCTGG	CACGACAGGT	TTCCCGACIG	GAAAGCGGGC	AGTACGCGCA	2100
ACGCAATTAA	TGIGAGTTAG	CTCCTCATTT	AGGCACCCCA	GGCTTTACAC	2150
TTTATGCTTC	CGGCTGGTAT	GTGTGTGGA	ATTGTGAGCG	GATAACAATT	2200
TCACACAGCA	AACAGCTATG	ACCATGATTA	CGAATTCCAG	CTCGGTACCC	2250
GGGGATCCIC	TAGAGTCGAC	CTGCAGGCAT	GCAAGCTTGC	ATGCCTCGAC	2300
GTGACGCTC	GGGGACTTGG	GTTCGCCATT	CTTTAGCGCG	CGTCGGGTCA	2350
CACAGCTTGG	CCACAATGIG	GTFTTTGTCA	AACGAAGATT	CTATGACGIG	2400
TTTAAAGTTT	AGGTCCAGTA	AAGCGCAAT	CTTTTTTAAC	CCTAGAAAGA	2450
TAGTCTGGGT	AAAATTGACG	CATGCATCT	TGAAATATTG	CTCTCTCTTT	2500
CTAAATAGCG	CGAATCCGTC	GCITGTGCTT	TAGGACATCT	CAGTCGCGGC	2550
TTGGAGCTCC	CGTGGGCGGT	GCITGTGCAAT	GGGTAAAGTG	TCACGTATTT	2600
TGAACATAAA	CGACCGGGTG	AGTCAAAATG	ACGCATGATT	ATCTTTTACG	2650
TGACTTTTAA	GATTTAACIC	ATAAGATAAT	TATATTGTTA	TTTCATGTTC	2700
TACTTACGIG	ATAACTTATT	ATATATATAT	TTTCTGTGTA	TAGATATCGT	2750
GACTAATAIA	TAATAAAATG	GGTAGTTCCT	TAGACGATGA	GCAATATCCIC	2800
TCTGCTCTTC	TGCAAAGCGA	TGACGAGCTT	GTGTGGTGGG	ATTCGTGACG	2850
TGAAATATCA	GATCAGGTAA	GTGAAGATGA	CGTCCAGAGC	GATACAGAAG	2900
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GAAATATTAG	ACGAACAAA	TGTTATTGAA	CAACCAGGTT	CTTCATTGGC	3000
TTCTAACAGA	ATCTTGACCT	TGCCACAGAG	GACTATTAGA	GGTAAGANTA	3050
AACATTGTTG	GTCAACTTCA	AAGTCCAGCA	GGGTAGCCG	AGTCTCTGCA	3100
CTGAACATTG	TCAGATCTCG	AGCTCAAGCT	TGGAATTCIG	CAGTCCAGCG	3150
TACCCGATCT	TGTGCGCGGA	ACGCAGCGAC	AGAGATTCCA	ATGTGTCCGT	3200
ATCTTTTACG	CTTTTGGCCT	TCAGTTCAG	ACGAAGCGAC	TGGCGATTCC	3250
CGTGTGGGGT	CTGCTTCAGG	GTCTGTGTGA	TTAGGGCGCG	CAGATCGCGG	3300
ATGGCGGTGG	CGCGGGAGGG	CACCTTCACC	TTGGCGTACG	GCTTGTCTGT	3350
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CAGTACTGTC	CAAAATCGAA	AATCGCGGAA	CCGTAGTGTG	ACCGTGGGGG	3450
GCTCTGGGAA	AATAAACCTT	TTTAGGTATA	TGGCCACACA	CGGGGAAAGC	3500
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TCAAAACCAA	TTGACATTIG	CAGCAGCAAG	TACAAGCAGA	AAGTAAAGCG	4300
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CTTAATGGCC	TTGCAGCACA	TCCCCCTTTC	GCCAGCTGGC	GTAATAGCGA	5300
AGAGGGGCGC	ACCGATGGCC	CTTCCCAACA	GTTCGGGTTC	ACTCTAGAGG	5350
ATCCCCGGGA	TCCACCGTTC	GCCACCATGG	TGAGCAAGCG	CGAGGAGCTG	5400

FIG. 6 c

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SUBSTITUTE SHEET (RULE 26)



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GCTGAAGGGC	ATCGACTTCA	AGGAGGACGG	CAACATCCIG	GGGCACAAGC	5800
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AAGAAGGGCA	TCAAGGTGAA	CTTCAAGATC	CGCCACAACA	TCGAGGAAGG	5900
CAGCGTGCAG	CTGGCCGACC	ACTACCAAGC	GAACACCCCC	ATCGGGGACG	5950
GGCCCGTGCT	GCTGCCCCGAC	AACCCTTACC	TGAGCACCCA	GTCCGCCCCIG	6000
AGCAAGAGCC	CCAACGAGAA	GCGGATCAC	ATGGTCCIGC	TGGAGTTCGT	6050
GAACGGGGCC	GGGATCACTC	TCGGCATGGA	CGAGCTGTAC	AAGTAAAGCG	6100
GGCGGACTC	TAGATCATAA	TCAGCCATAC	CACATTTGTA	GAGGTTTTCAC	6150
TTGCTTTTAA	AAACCTCCCA	CACCTCCCC	TGAACCTGAA	ACATAAAATG	6200
AATGCAATTG	TTGTTGTATA	CTGTGTTTAT	GCAGCTTATA	ATGGTTTACAA	6250
ATAAAGCAAT	AGCATCACAA	ATTTCACAAA	TAAAGCATTT	TTTTCACCTG	6300
ATTCATAGTG	TGGTTTGTCC	AAACCTATCA	ATGTATCTTA	AGCGGTAAAT	6350
TGTAAGGGTT	AATATTTTGT	TAAATTTCCG	GTAAATTTT	TGTTAAATCA	6400
GCTCATTTTT	TAACCAATAG	GGCGAATCG	GCAAAATCCC	TTATAAATCA	6450
AAAGCAATAG	CCGAGATAGG	GTTGAGTGT	GTTCAGTTT	CGAACAAGAG	6500
TCCACTATTA	AAGAAGGAG	ACTCCAAGT	CAAAGGGCGA	AAAACCGCTT	6550
ATCAGGGCGA	TGGCCCACTA	CGTGAACAT	CACCTAATC	AAGTTTTTTG	6600
GGGTCCAGGT	GCCGTAAAGC	ACTAAATCGG	AACCTTAAAG	GGAGCCCCCG	6650
ATTTAGAGCT	TGACGGGGAA	AGCCGGGGAA	CGTGGCGAGA	AAGGAAGGGA	6700
AGAAAGCGAA	AGGAGGGGGC	GCTAGGGGGC	TGGCAAGTGT	AGCGGTACAG	6750
CTGCGGGTAA	CCACCACACC	CGCCGGGCTT	AATGCGGGGC	TACAGGGGGC	6800
GTCAGGTGGC	ACTTTTCCGG	GAAATGTGGG	CGTAACCCCT	ATTTGTTTAT	6850
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TAAATGCTTC	AATAATATTG	AAAAAGGAAG	AGTCCCTGAG	CGGAAAGAAC	6950
CAGCTGTGGA	ATGTGTGTCA	GTTAGGGTGT	GGAAGTCCC	CAGGCTCCCC	7000
AGCAGGCAGA	AGTATGCAAA	GCATGCATCT	CAATTAGTCA	GCAACCAAGT	7050
GTCGAAAGTC	CCAGGCTCC	CCAGCAGGCA	GAAGTATGCA	AAGCATGCCAT	7100
CTCAATTAGT	CAGCAACCAT	AGTCCCGGCC	CTAATCCGC	CCATCCCGCC	7150
CCTAACTCCG	CCAGTTCGG	CCATTTCTCC	GCCCCATGGC	TGACTAATTT	7200

FIG. 6d

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AAACAAAGCC	GAGATACCGG	AAGTACIGAA	AAACAGTCGC	TCCAGGCCAG	7350
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TTTGAAGAGA	TATTTGCGCG	ATAATATCTC	TAATATTTTG	CCAAATGAAG	7800
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GTCAAAAAA	TGCAAAAAG	TTATTTGTCG	AGACATAAT	ATTGATATGT	7950
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GTCACATAT	GATTATCTTT	CTAGGGTTAA	ATAATAGTTT	CTAATTTTTT	8350
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GCCCGACGIG	TTGGCTAAAA	TTATTAATTC	AACCTGIGIT	ATAGTCACCG	8900
ATTTGGCGTC	CAACGTGTTT	CTCAAAAAGT	TGAAGACCAA	CAAGTTTACG	8950
GACACTATTA	ATTATTGAT	TTTGGCCAC	TTCATTTTGT	GGCATCACAA	9000

FIG. 6e

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TCCCCCTTTC	GCCAGCTGGC	GTAATAGCGA	AGAGGCCCCG	ACCGATCGCC	9150
CTTCCCAACA	GTTCGCCAGC	CTGATGGCG	AATGGGGGCT	GATGCGGTAT	9200
TTTCTCTTA	CGCATCTGIG	CGGTATTTCA	CACCGCATAT	GGTGCATCT	9250
CAGTACAATC	TGCTCTGATG	CGCATAGTT	AAGCCAGCCC	CGACACCCGC	9300
CAACACCCGC	TGACCGGCCC	TGACGGGCTT	GTCTGCTCCC	GGCATCCGCT	9350
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ACCGTCAATCA	CCGAAACGCG	CGA			9423

FIG. 6 f

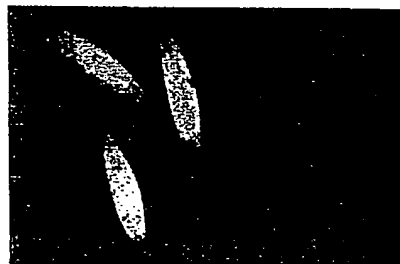


FIG. 7a

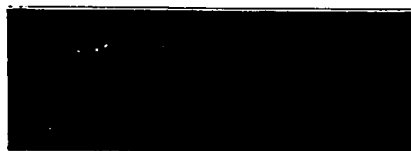


FIG. 7b



FIG. 7c



FIG. 7d

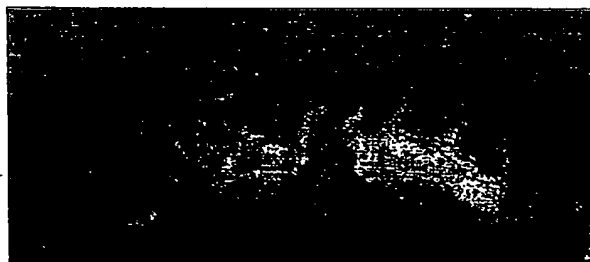


FIG. 7e

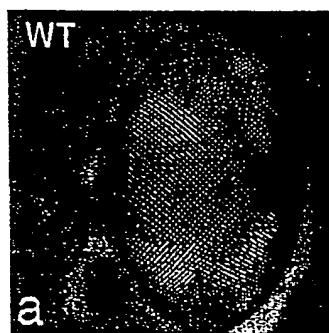


FIG. 8a

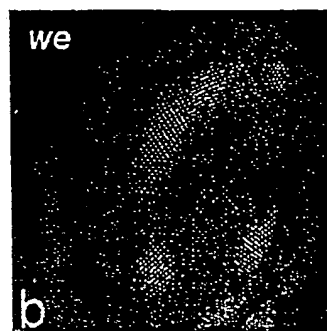


FIG. 8b

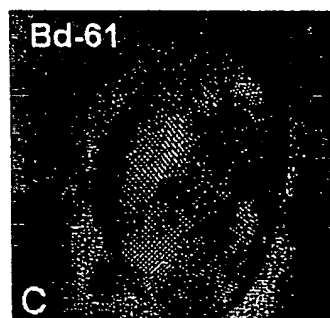


FIG. 8c

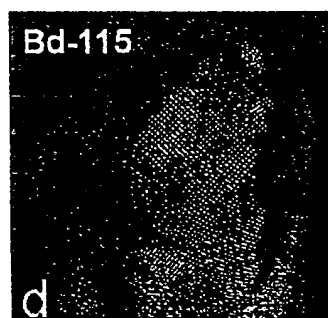


FIG. 8d

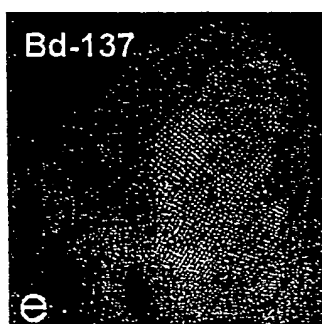


FIG. 8e



FIG. 9a

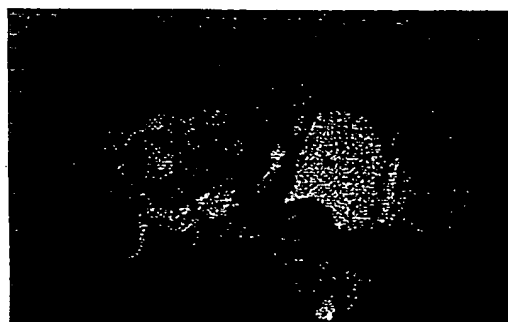


FIG. 9b

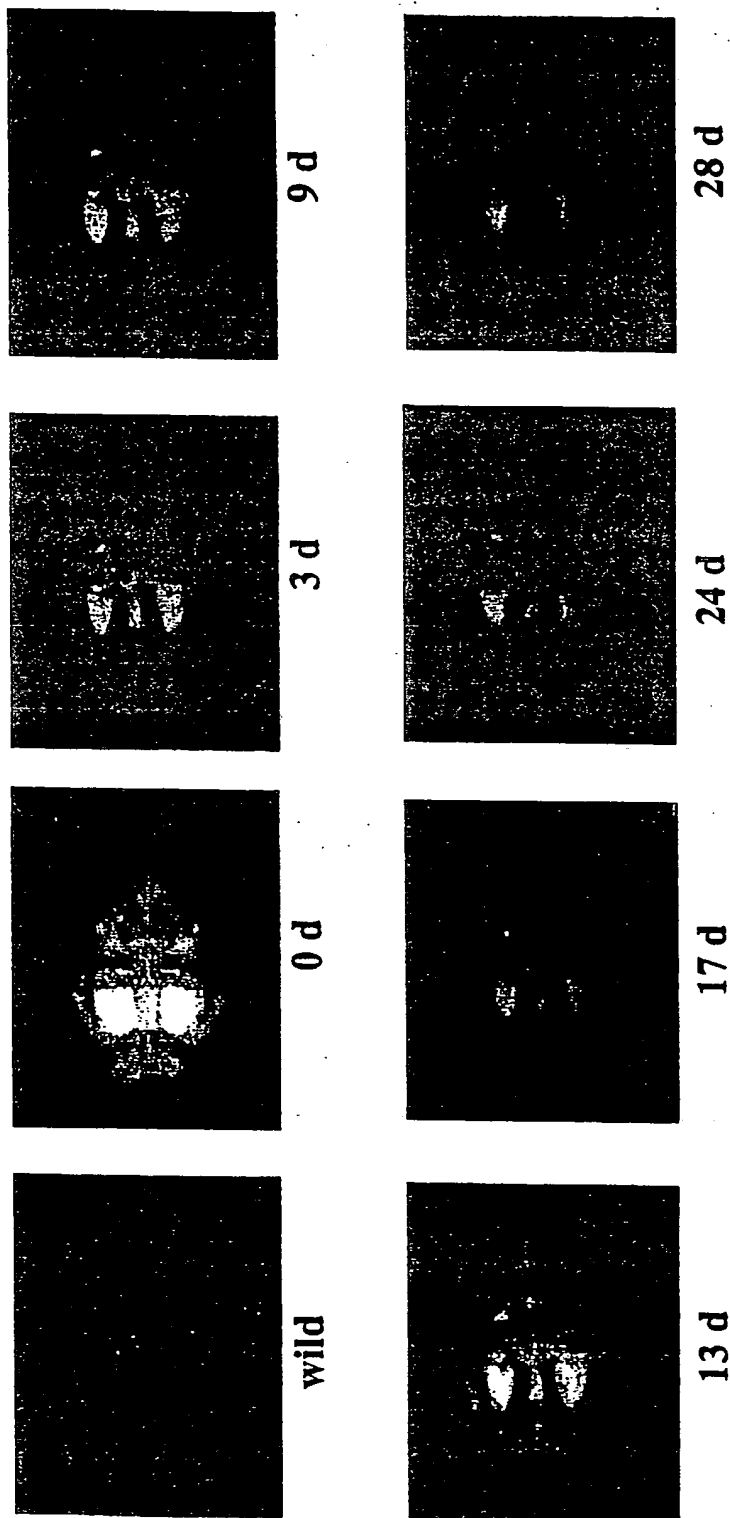


FIG. 10



## SEQUENCE LISTING

<110> Handler, Alfred M.

<120> PiggyBac Transformation System

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/22433

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :C12N 15/00, 15/63; A01K 67/00, 67/027

US CL :435/320.1, 455; 800/13, 14, 19, 20

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 455; 800/13, 14, 19, 20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, CAPLUS, BIOSIS, SCISEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HANDLER et al., The Lepidopteran Transposon Vector, piggybac, Mediates germ-Line Transformation in the Mediterranean Fruit Fly. Proc. Natl. Acad. Sci., USA. June 1998, Vol. 95, pp. 7520-7525, especially pages 7520, 7521.	1-9
Y	CORMACK et al. ACS-Optimized Mutants of the Green Fluorescent Protein (GFP). Science. 1996, Vol. 173, pp. 33-38, especially pages, 33, 38.	1-9
Y	DAVIS et al. A Nuclear GFP That Marks Nuclei in Living Drosophila Embryos; Maternal Supply Overcomes a Delay in the Appearance of Zygotic Fluorescence. Developmental Biology. 1995, Vol. 170, pp. 726-729, especially pages, 726, 729.	1-9

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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 22 SEPTEMBER 2000	Date of mailing of the international search report 02 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Shin-Lin Chien</i> SHIN-LIN CHIEN Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/22433

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	O'BROCHTA et al. Transposable Elements and Gene Transformation in Non-Drosophilid Insects, Insect Biochem. Molec. Biol., 1996, Vol. 26, Nos. 8-9, pp. 739-753, especially pages 739, 740, 749, 750.	7-9
A	ASHBURNER et al. Prospects for the Genetic Transformation of Arthropods. Insect Molecular Biology. 1998, Vol. 7, No. 3, pp. 201-213, especially pages 201, 205.	7-9

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